DNA Concentrations in the Human Cerebellum. Computation from Kinetics of Deoxyribose Extraction in Hot Acid

DANIEL N. SLATKIN ^a and SØREN LØVTRUP ^b

^a Medical Department, Brookhaven National Laboratory, Upton, New York, U.S.A. and

^b Department of Zoophysiology, University of Umeå, S-Umeå, Sweden

DNA can be measured in mammalian tissues by extracting deoxyribose from unfixed, lyophilized tissue specimens with 0.5 N perchloric acid at 90 °C. Deoxyribose concentrations in the extract are determined photometrically by reaction with diphenylamine. Inevitably, some deoxyribose is destroyed during exposure to the hot acid. A computer program has been written which corrects photometric absorbance data for such loss of deoxyribose. When extrapolated to infinite duration of extraction, the corrected absorbances yield a measure of the DNA content of the specimen.

This method was used to estimate DNA concentrations in human cerebellar cortex and white matter. The results are discussed in relation to stable carbon isotope ratios of human cerebellar DNA.

Several photometric methods for estimating tissue DNA are based on measurements of extractable deoxyribose, because almost all deoxyribose in animal tissues exists as the pentose moiety of DNA.¹ Deoxyribose can be almost completely extracted from mammalian tissues within a few hours using 0.5 N perchloric acid at 90 °C. To compensate for the concomitant partial destruction of deoxyribose during its exposure to hot acid, Løvtrup and Roos devised a reiterative graphical procedure (LRP) for extrapolating the corrected absorbance, A_o^* , from the sequence of absorbances observed after the reaction of diphenylamine with sequential aliquots of the extract.²⁻⁴ They found that A_o^* , the corrected absorbance which would have been observed after prolonged extraction had there been no

concomitant destruction of deoxyribose, correlated well with independent microbiological assays of tissue DNA.

Although other techniques are available for determining DNA in tissue, photometric methods based on the extraction of deoxyribose from tissue powder by hot acid,⁵⁻⁷ the so-called Schneider procedure, are convenient and are used widely. It has been found that DNA concentrations measured by such extractions vary in accuracy with the type of tissue being analyzed and with the physiological state of cells in a specimen, apparently due to differences in the extractability of deoxyribose or to variable rates of deoxyribose destruction by hot acid.⁸⁻¹¹ In 1966, Munro and Fleck¹² suggested that automated analysis may restore the attractiveness of the Schneider procedure in spite of inherent errors in extraction. The computation method described in this communication fulfills Munro and Fleck's criterion of full automation of data analysis, while taking into account not only the kinetics of deoxyribose extraction and destruction but also the presence of acid-resistant interfering substances in the extract. Although the principle of the calculation is straightforward, a small computer is required to carry it out.

THEORY

In the LRP each absorbance was corrected by adding the estimated loss of absorbance attributable to prior destruction of deoxyribose in the hot acid extract. Program CORABS (Tables 1 and 2) obtains the corrected absorbance, A_o^* , using a

similar technique. Correction is discontinuous, as in the LRP, until the time of maximum absorbance, t_m . Thereafter, correction is continuous by integration of a function which fits absorbance values in the range $t \geq t_m$, where t is the duration of extraction. Suppose

$$A(t) = K + A_u(t) \quad (1)$$

where $A(t)$ is the absorbance observed after t min of extraction, $A_u(t)$ is the portion of observed absorbance attributable to deoxyribose, and K is the absorbance attributable to acid-resistant interfering substances. Assume that, for $t > t_0$, extraction and destruction kinetics are exponential. That is, if

$$t' = t - t_0 \quad (2)$$

$$dA_u(t')/dt' = k_u D e^{-k_u t'} - k_e A_u(t') \quad (3)$$

where k_u and k_e are the apparent time constants for extraction and destruction of deoxyribose, respectively, and D is the tissue deoxyribose pool at $t' = 0$. Such kinetic behavior implies

$$A(t') = K + S_1 e^{-k_e t'} - S_2 e^{-k_u t'} \quad (4)$$

where S_1 and S_2 are constants of integration.

Actually, absorbance values are found to fit similar equations of the form

$$A(t - t_0) = V + S e^{-k_e(t-t_0)} - S e^{-k_u(t-t_0)} \quad (5)$$

where $t \geq t_m$ and V , S , k_u , k_e and t_0 are empirical constants. Thus, if A_m^* is the absorbance at $t = t_m$ corrected by the LRP for deoxyribose loss prior to t_m ,

$$A_o^* = A_m^* + \int_{t'_m}^{\infty} (A(t') - V) \cdot k_e \cdot dt' \quad (6)$$

where

$$t'_m = t_m - t_0 \quad (7)$$

These equations yield A_o^* , the absorbance corrected for loss of deoxyribose and extrapolated to infinite duration of extraction:

$$A_o^* = A_m^* + k_e \cdot S \cdot (e^{-k_e t'_m}/k_e - e^{-k_u t'_m}/k_u) \quad (8)$$

Eqn. (5) is similar to the following equation of the LRP.

$$A(t) = A_{\infty} + (A_0 - A_{\infty}) \times 10^{-kt} \quad (9)$$

V is the analogue of A_{∞} and S is the analogue of $(A_0 - A_{\infty})$. The analogue of k is k_e where

$$k_e = k(\ln 10) \quad (10)$$

Since eqn. (9) has no parameter analogous to k_u , it cannot fit absorbances at or near the maximum absorbance.

COMPUTER PROGRAM

Program CORABS determines empirical values of t_0 , V , S , k_e and k_u which minimize the deviation of observed absorbance values from isochronous absorbance values on the fitted curve (eqn. (5)). It is postulated that the probability of relating absorbance values with deoxyribose concentrations decreases with time exponentially, with the same time constant as that of deoxyribose destruction. Thus, in line 150 of the curve-fitting algorithm of CORABS, each absorbance point, $A(t)$, is assigned the statistical weight $e^{-k_e t} \cdot |A(t) - K|$.

The calculation of A_m^* in eqn. (8) is based on the LRP. In the LRP, the average absorbance between the start of extraction and t_1 , the time of the first observed absorbance, is estimated as $0.75 A(t_1)$ if $t_1 = t_m$ and as $0.50 A(t_1)$ if $t_1 \neq t_m$. A quantitative basis for such approximations was developed by observing that the initial rapid increase in absorbance is roughly exponential. Then, if

$$x = (A(t_1) - K)/(A(t_m) - K) \quad (11)$$

it can be shown that the ratio to $A(t_1) - K$ of the average net absorbance prior to t_1 is

$$y \approx x^{-1} + (\ln(1-x))^{-1} \quad (12)$$

In the range $0.90 < x < 0.99$, y varies from 0.68 to 0.79, and averages 0.72, the factor used in line 220 of CORABS for $t_1 = t_m$. This is close to the factor 0.75 used in the LRP. If $t_1 \neq t_m$, eqns. (11) and (12) are used to estimate the average absorbance in the range $0 < t < t_1$. In CORABS,

absorbances at or below $A(t_m)/5$ are automatically neglected in the calculation of A_o^* . This minimizes errors in A_o^* which otherwise could be attributed to acid-resistant interfering substances extracted slowly from tissue powder.

The curve-fitting algorithm of CORABS makes use of the following relationships among k_u , k_e , t_m , and t_o . If

$$f = (t_m - t_o)/w \quad (13)$$

where w min is the time interval between aliquot samples of the extract, and if

$$g = k_u/k_e \quad (14)$$

it may be shown from the first derivative of eqn. (5) that

$$\ln g = wfk_e(g-1) \quad (15)$$

The solution of eqn. (15) is

$$g = \lim [1 + \ln(1 + \ln(1 + \ln \dots (1 + \ln(1 + \ln g_o/(wfk_e)) \dots))] \quad (16)$$

where g_o is any real number greater than one. Eqn. (16) is used in line 120 of CORABS. Eqns. (13) through (16) are used in CORABS to calculate provisional values of k_u from tentative values of t_o and k_e , as indicated by the first command of line 130 in CORABS. Provisional values of k_e are calculated from absorbance values at $t \gg t_m$ using Guggenheim's algorithm,¹³ as in the LRP.

The particular version of program CORABS displayed in Table 1 is written in a BASIC computer language specified for the Casio FX-702P pocket computer.¹⁴ In addition to standard BASIC functions, the following special statistical functions of this portable (186 g) minicomputer are used in CORABS.

STAT x,y assigns the statistical weight y to the number x in a series of numbers during calculation of the standard deviation.

Table 1. List of program CORABS.

```

10 X=0:Z=9E99:INP"W",W,"P",P:FOR I=1 TO P:PRT" A";W*I;
20 INP A(I):PRT"A";W*I;"=";A(I);:IF A(I)>X;X=A(I):M=I
30 NEXT I:L=P:FOR I=M+1 TO P:IF A(I)>= .7*A(M);Q=I:PRT"!";
40 IF A(I)<=A(M)/5;L=I-1:I=P
50 NEXT I:FOR N=M TO Q:PRT"WAIT FOR A=A0";Q-N+1;"/";Q-M+1;
60 J=INT((L-N)/2):G=30:H=9E99:F=0:Y=0
70 SAC:FOR I=0 TO L-N-J:X=A(N+I)-A(N+I+J):IF X<0 THEN 90
80 STAT W*(N+I),LN X
90 NEXT I:B=LRB:X=EXP(B*W):C=EXP LRA/(1-X+J):SAC
100 FOR I=0 TO L-N:STAT A(N+I)-C*X+(N+I):NEXT I:K=MX:J=.4
110 F=F+J:IF F<0 THEN 110
120 X=G:G=1-LN X/(F*W*B):IF ABS(X-G)>.005 THEN 120
130 O=G*B:D=C*EXP(B*W*(M-F)):SAC:FOR I=M TO L
140 X=W*(I-M+F):X=EXP(B*X)-EXP(O*X)
150 STAT((K+D*X)/A(I)-1)^2;ABS(A(I)-K)*EXP(B*W*I)
160 NEXT I:IF F=Y THEN 210
170 IF MX<H;H=MX:G=30:GOTO 110
180 IF ABS(1-MX/H)<1E-04 THEN 200
190 F=F-5*J/2:J=J/2:G=30:H=9E99:GOTO 110
200 F=F-3*J/2:Y=F+J:G=30:GOTO 110
210 A(0)=0:A(L+1)=0:FOR I=1 TO M:IF M*I>1 THEN 230
220 A(L+2)=A(1)-B*W*.72*ABS(A(1)-K):GOTO 270
230 IF I#1 THEN 260
240 Y=ABS((A(1)-K)/(A(M)-K)):Y=1/Y+(LN(1-Y))^(-1)
250 A(L+2)=A(1)-B*W*Y*ABS(A(1)-K):GOTO 270
260 A(L+I+1)=A(L+I)+A(I)-A(I-1)-B*W*(A(I)+A(I-1))/2
270 NEXT I:T=A(L+M+1)-B*D*(EXP(O*W*F)/O-EXP(B*W*F)/B)
280 IF MX<Z;Z=MX:E=B:V=K:U=O:R=W*(M-F):S=D:P=A(L+M+1):A=T
290 NEXT N:PRT" A0";A:END

```

MX calculates the weighted arithmetic mean of data entered during standard deviation analysis.

STAT x,y;z assigns the statistical weight *z* to the point (*x,y*) in a series of points during calculation of the regression line through those points.

LRA and *LRB* calculate the *y*-intercept and the slope of the regression line, respectively.

SAC clears the statistical processing registers.

The commands, *STAT*, *MX*, *LRA*, *LRB*, and *SAC* can be replaced by subroutines if *CORABS* is modified for a computer which does not happen to use such statistical functions directly. When running program *CORABS*, the time interval in minutes between removal of aliquots

of the extract and the total number of such aliquots are entered and stored in registers *W* and *P*, respectively. The 600 nm absorbances determined from these aliquots are entered sequentially and stored in the array *A*(1), *A*(2), ..., *A*(*P*). These absorbance values correspond to *W*, *2W*, ..., and *PW* minutes of extraction, respectively. A_o^* is then computed, stored in register *A*, and displayed automatically. Registers *B* through *Z* are used for other parameters of *CORABS*, as shown in Table 2.

The following separate three-command program can be used to generate points on the fitted curve, eqn. (5).

```
1 INP "T", T:PRT V+S*(EXP(E*(T-R))-EXP(U*(T-R))):GOTO 1
```

Table 2. Parameters of program *CORABS*.

A	Computed value of A_o^* , eqn. (8)
B	Provisional values of $-k_e$
C	Intermediate parameter, analogous to $A_o - A_\infty$ of the original Løvtrup-Roos procedure
D	Provisional values of <i>S</i>
E	Computed value of $-k_e$
F	Intermediate parameter <i>f</i> , eqn. (13)
G	Intermediate parameter <i>g</i> , eqns. (14)–(16)
H	Intermediate variable
I	Integral variable
J	Intermediate parameter; <i>W</i> * <i>J</i> corresponds to τ of Guggenheim's algorithm, Ref. 13
K	Provisional values of <i>V</i>
L	Array integer for the last observed absorbance greater than $A(t_m)/5$
M	Array integer for the maximum observed absorbance, $A(t_m)$
N	Integral variable
O	Provisional values of $-k_u$
P	Number of observed absorbance points; finally, computed value of A_m^*
Q	Array integer for the last observed absorbance greater than or equal to $0.7A(t_m)$
R	Computed value of t_o , eqn. (5)
S	Computed value of <i>S</i> , eqn. (5)
T	Provisional values of A_o^*
U	Computed value of $-k_u$
V	Computed value of <i>V</i> , eqn. (5)
W	Constant time interval between aliquots of acid extract, minutes
X	Intermediate variable
Y	Intermediate variable
Z	Weighted mean square fractional deviation of absorbance points from the fitted curve, eqn. (5)

Examples of fitted curves together with the absorbance points from which they are derived are shown in Fig. 1, where absorbance data are from case No. 1.

EXPERIMENTAL

Lateral lobes of four normal human cerebella were removed *post-mortem*, then frozen at -70°C pending DNA analysis. Information concerning the autopsies is shown in Table 5. Each cerebellum was normal, and the decedents showed no evidence of primary neurological disease. Cadavers were refrigerated prior to the autopsy.

Each cerebellar specimen was divided into white matter and cortical portions by macroscopic dissection, and the portions were lyophilized separately. Weighed portions of these tissue powders and known quantities of calf thymus DNA were treated concurrently with 5 % perchloric acid at 90°C , as described previously.²⁻⁴ Aliquots (1 ml) of the extracts were removed every 5 min. The aliquots were treated with diphenylamine, and the resulting 600 nm absorbance readings were tabulated for entry into the array *A*(1), *A*(2), ..., *A*(*P*). The results of processing these data with program *CORABS* are shown in Tables 3 and 4.

DISCUSSION

Only a few investigators^{15,16} have reported using the Løvtrup-Roos correction procedure, presumably because the original procedure is difficult. Since the computation of A_o^* is now

Fig. 1. Absorbance data and fitted curves for case No. 1. The upper curve (open circles, cerebellar cortex) corresponds to the ordinate on the right (scale: zero to 1.20). The middle (closed circles, cerebellar white matter) and lower (small x's, DNA control) curves correspond to the ordinate on the left (scale: zero to 0.30).

automated, the Løvtrup-Roos correction should be more easily applied.

The computer program presented here (Tables 1 and 2) is based on the original Løvtrup-Roos procedure (LRP). It uses an algorithm which fits an empirical kinetic equation to absorbance values beginning at the time of extraction which yields maximum absorbance. In the LRP, A_0^* is obtained by extrapolation from absorbances observed during the first few hours of extraction. The program presented here uses a formula to calculate A_0^* by extrapolation to infinite duration of extraction with continuous compensation for destruction of deoxyribose.

Tissues analyzed were *post-mortem* specimens of human cerebellar cortex and cerebellar white matter. It can be calculated from the results of analyses listed in Tables 3 and 4 that average concentrations of DNA in cerebellar cortex and white matter are $0.72 \pm 0.02 \mu\text{g}$ per mg dry tissue and $0.05 \pm 0.01 \mu\text{g}$ DNA per mg dry tissue, respectively. It was also found that the average concentration of DNA in the macroscopically dissectable human cerebellar leptomeninges and blood vessels is less than $0.20 \mu\text{g}$ per mg dry weight. Comparison of Tables 4 and 5 shows no correlation between age, time interval between death and autopsy, and cerebellar DNA concen-

Table 3. Results obtained using the program CORABS.

Case No.	Specimen	A_m^*	S	V	t_0	k_u	$k_e \times 10^2$
1	Cerebellar cortex	1.260	1.285	0.108	-1.72	0.4921	2.0736
1	Cerebellar white matter	0.419	0.354	0.081	8.61	0.1990	2.8571
1	Standard DNA	0.300	0.287	0.032	-0.28	0.6879	2.0231
2	Cerebellar cortex	1.237	1.045	0.098	6.70	1.2884	1.9652
2	Cerebellar white matter	0.510	0.625	0.055	-2.84	0.1786	2.4805
2	Standard DNA	0.301	0.283	0.032	0.38	0.8203	2.0418
3	Cerebellar cortex	1.335	0.751	0.149	20.00	4137.7	2.2501
3	Cerebellar white matter	0.377	0.292	0.059	9.98	0.7135	2.2269
3	Standard DNA	0.312	0.365	0.044	-7.32	0.2015	2.2103
4	Cerebellar cortex	1.109	1.649	-0.390	-0.59	0.3318	1.1099
4	Cerebellar white matter	0.419	0.366	0.005	14.61	0.6162	2.5520
4	Standard DNA	0.320	0.263	0.032	5.00	8808.5	2.1936

Table 4. Results obtained using the program CORABS.

Case No.	Specimen	Dry weight	Corrected absorbance A_{260}^*	DNA concentration $\mu\text{g}/\text{mg}$ dry tissue
1	Cerebellar cortex	209.8 mg	2.376	0.702
1	Cerebellar white matter	895.2 mg	0.669	0.046
1	Standard DNA	34.6 μg	0.558	—
2	Cerebellar cortex	185.0 mg	2.216	0.741
2	Cerebellar white matter	904.8 mg	0.955	0.065
2	Standard DNA	34.6 μg	0.559	—
3	Cerebellar cortex	170.9 mg	2.086	0.719
3	Cerebellar white matter	929.5 mg	0.638	0.040
3	Standard DNA	34.6 μg	0.587	—
4	Cerebellar cortex	210.7 mg	2.574	0.725
4	Cerebellar white matter	914.7 mg	0.737	0.048
4	Standard DNA	34.6 μg	0.583	—

Table 5. Post-mortem diagnoses.

Case No.	Age, sex	Post-mortem interval to autopsy	Post-mortem diagnoses ^a
1	64 yr, male	<10 hr	Anaplastic carcinoma of the urinary bladder with local and systemic metastases. Microscopic metastases in the cerebral dura and the superior sagittal sinus; brain otherwise normal. Left hydronephrosis. Recent peptic ulcer of the duodenum.
2	60 yr, male	17 hr	Severe panacinar emphysema. Severe right ventricular hypertrophy. Ascites, peripheral edema, bilateral hydrothorax and pericardial effusion. Fibrinous pericarditis. Chronic passive congestion of the liver. Multiple organizing pulmonary arterial thrombi, probably embolic. Tracheostomy. Large sacral decubitus ulcer. Small left parietal meningioma and two small, organized cerebral infarcts, both asymptomatic; brain otherwise normal.
3	73 yr, male	8 hr	Squamous cell carcinoma of the lung with local and systemic metastases. Severe coronary artery atherosclerosis with left ventricular myocardial scarring and hypertrophy. Acute purulent bronchopneumonia. Brain normal.
4	62 yr, male	27 hr	Squamous cell carcinoma of the esophagus with local and systemic metastases. Esophageal perforation with focal subacute mediastinitis. Two small carcinoid tumors of the ileum, asymptomatic. Acute bronchopneumonia and pulmonary edema. Brain normal.

^a D. N. Slatkin and N. Peress.

trations.

It has been estimated that the convoluted cerebellar cortex occupies 90 % of the volume of the cerebellum.¹⁷ Assuming conservatively that not more than 5 % of the cerebellum is occupied by leptomeninges and blood vessels, it is inferred that the non-vascular moiety of the human cerebellar cortex constitutes about 85 % of the cerebellum as a whole. Since 95 % of cerebellar cortical nuclei are in the granular layer of the cortex¹⁸ and since, in the mammal, over 99 % of granular layer nuclei are neuronal,¹⁹ it follows that approximately 94 % of all cerebellar cortical nuclei are neuronal. Using the values of DNA concentrations presented here, it is calculated that 92 % of whole cerebellar DNA is situated in neurons of the cerebellar cortex. These calculations are in accord with observations on nuclear fractions of whole cerebellar homogenates from dogs and rats, which show that about 90 % of cerebellar nuclei are neuronal.^{20,21}

These measurements of cerebellar DNA lend support to the postulate that there is significant turnover of cerebellar neuronal DNA during the human lifetime.²² That postulate is based on stable carbon isotope ratio measurements on the carbon moiety of DNA isolated from unfractionated homogenates of human cerebellar tissue from which only minor portions of the cerebellar white matter and leptomeninges had been removed prior to homogenization for DNA extraction. The foregoing calculations indicate that 90–92 % of such carbon is derived from cerebellar neuronal DNA. Human tissue ¹³C/¹²C ratios are significantly lower in Europeans than in Americans.²³ Thus, the finding that ¹³C/¹²C ratios in whole cerebellar DNA from European-born Americans tend to approach ratios which are typical of American tissues is interpreted as evidence that a substantial proportion of neuronal DNA in the cerebellum may undergo slow turnover during adult life.

Acknowledgements. The authors thank Dr. N. Peress for neuropathological examinations, Mrs. Peg Micca and Mrs. Mary Wigger for skilled technical and secretarial assistance, and Drs. S. L. Commerford, H. Løvtrup-Rein, E. A. Popenoe, J. S. Robertson and L. Sokoloff for helpful comments. This work was supported in part by the U.S. Department of Energy.

REFERENCES

1. Munro, H. N. and Fleck, A. *Methods Biochem. Anal.* 14 (1966) 113.
2. Løvtrup, S. and Roos, K. *Biochim. Biophys. Acta* 53 (1961) 1.
3. Løvtrup, S. *Acta Biochim. Pol.* 9 (1962) 411.
4. Løvtrup, S. and Roos, K. *Biochim. Biophys. Acta* 68 (1963) 425.
5. Burton, K. *Methods Enzymol.* 12 (1968) 163.
6. Webb, J. M. and Levy, H. B. *J. Biol. Chem.* 213 (1955) 107.
7. Ceriotti, G. *J. Biol. Chem.* 198 (1952) 297.
8. Holm, B. *Biochim. Biophys. Acta* 119 (1966) 647.
9. Barkowski, T. and Sikorska, K. *Acta Biochim. Pol.* 11 (1964) 451.
10. Santen, R. J. and Agranoff, B. W. *Biochim. Biophys. Acta* 72 (1963) 251.
11. Hutchinson, W. C., Downie, E. D. and Munro, H. N. *Biochim. Biophys. Acta* 55 (1962) 561.
12. Munro, H. N. and Fleck, A. *Analyst* 91 (1966) 78.
13. Guggenheim, E. A. *Philos. Mag. Ser. 7*, 2 (1926) 538.
14. Casio Inc., 15, Gardner Road, Fairfield, New Jersey 07006, U.S.A.
15. Martinsson, A., Sunzel, H. and Hood, B. *Acta Med. Scand.* 173 (1963) 745.
16. Baldwin, R. L. and Milligan, L. P. *J. Biol. Chem.* 241 (1966) 2058.
17. Smolyaninov, V. V. In Gelfand, I. M. et al., Eds., *Models of the Structural-Functional Organization of Certain Biological Systems*, Moscow 1966; Engl. transl. Beard, C. R., MIT Press, Cambridge, Massachusetts 1971, pp. 252–325.
18. Blinkov, S. M. and Glezer, I. I. *The Human Brain in Figures and Tables*, Basic Books, New York 1968.
19. Palkovits, M., Magyar, P. and Szentagothai, J. *Brain Res.* 32 (1971) 15.
20. Wheeler, K. T. and Lett, J. T. *Radiat. Res.* 52 (1972) 59.
21. Kuenzle, C. C., Knüsel, A. and Schümperli, D. *Methods Cell Biol.* 15 (1977) 89.
22. Slatkin, D. N., Irsa, A. P. and Friedman, L. In Klein, E. R. and Klein, P. D., Eds., *Stable Isotopes*, Academic, New York 1979, p. 239.
23. Gaffney, J. S., Irsa, A. P., Friedman, L. and Slatkin, D. N. *Biomed. Mass Spectrom.* 5 (1978) 495.

Received July 16, 1982.